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Tagging the next generation: validation of trans-generational chemical tagging for an endangered fish

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Abstract In this study we validated marking offspring through peritoneal injection of ripe females using two concentrations of strontium (strontium chloride hexahydrate). Larvae from treatments were monitored for condition morphometrics and tested for chemical mark incorporation in their otoliths via laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) to quantify the strontium concentration (Sr/Ca) and laser ablation multi-collector inductively coupled plasma mass spectrometry (LA-MC-ICPMS) to measure the strontium isotope ratios (^{87}Sr:^{86}Sr) of otoliths. Otolith strontium concentrations and strontium isotopes ratios were elevated in the high-concentration treatment, while the low-concentration and control treatments were not significantly different from each other. Larval size and eye diameter at hatch were similar among treatments; however, yolk and oil globule diameters were significantly reduced in the high-concentration treatment. Moreover, growth rates after 60 days post-hatch were significantly reduced in the high-concentration treatment relative to the low-concentration and control treatments, suggesting trans-generational tagging can have deleterious effects on offspring. Our study provides evidence for the efficacy of artificially marking offspring via injection of strontium into ripe females and could provide new tools for managing endangered fish populations; however, careful consideration of chemical concentrations and dosages may be required prior to its application in a fisheries management experiment.

Keywords Otolith · Microchemistry · Trans-generational chemical tag · Delta smelt · Laser ablation

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Introduction

Manually tagging fish is a standard technique used to study fish movements, estimate population abundance, or monitor stock enhancement programs. However, this kind of marking is often limited to

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larger juvenile to adult stage fishes (Nielsen 1992). In contrast, larval to small juvenile stage fishes have been marked through immersion in fluorescent dyes (Tsukamoto 1985; Tsukamoto et al. 1989; Secor et al. 1991; Jones et al. 1999, 2005), solutions containing altered trace element concentrations (Brothers 1990; Schroeder et al. 1995), or artificially enriched isotopes that are incorporated into hard-parts (Munro et al. 2008). With the development and expansion of laser ablation techniques, the use of otolith micro-chemistry as a natural or artificial tag has had an increased role in understanding connectivity among reef fish populations (Thorrold et al. 2002; Swearer et al. 2002; Almany et al. 2007), migration histories (Secor et al. 1995, 2001; Elsdon and Gillanders 2003), and natal origins of mixed stock populations (Campana et al. 2000; Secor et al. 2001).

Recent studies have used in-vitro methods to chemically mark the otolith cores of offspring through direct injections of ripe females with elevated concentrations of strontium chloride or enriched isotopes of barium (Thorrold et al. 2006; Almany et al. 2007; Williamson et al. 2009; Kuroki et al. 2010; Shippentower et al. 2011). This technique allows for efficient marking of a large number of offspring in a single effort. Moreover, injections of strontium chloride and enriched barium isotopes have not been shown to have adverse effects on adult fish or their offspring, and have exhibited high success rates in marking the otoliths of offspring (Williamson et al. 2009; Shippentower et al. 2011).

The delta smelt is a small estuarine fish, endemic to the upper San Francisco Bay-Delta, CA. It was federal and state listed as threatened species in 1993 and state up-listed as endangered in 2009.

While rare, the use of artificial propagation of endangered species to enhance wild populations can serve as an important conservation strategy. Paramount to any artificial population enhancement project, a means by which artificially produced individuals can be tagged and tracked is necessary. In this study, we validate the technique of in-vitro chemical marking of delta smelt progeny through peritoneal injections of ripe females with strontium chloride hexahydrate at two concentrations. Offspring of treated females were examined for elevated strontium concentrations in otoliths, altered strontium isotope ratios (^{87}Sr:^{86}Sr), and deleterious effects of exposure on size-at-hatch, oil and yolk volume and eye development.

Methods

Laboratory validation of trans-generational chemical markings

Trans-generational marking of fecund, F1 generation, cultured delta smelt and rearing of offspring were both conducted at the UC Davis Fish Conservation and Culture Laboratory, in Byron, CA. Three groups of anesthetized fish, chosen based on external examination of their reproductive stage, were respectively injected with: 2 μL/g (wet mass) of an isotonic saline solution (as control), a low-concentration (15 μg/L) and a high-concentration (30 μg/L) of strontium chloride hexahydrate, into the peritoneal cavity. In-vitro fertilization was accomplished through manual expression of gametes in a subset of three fish from each treatment several days post injection. Embryos were incubated in flow-through, column-style incubators. Upon hatch, larvae were moved to 70 L black tanks and fed a diet of rotifers and brine shrimp *Artemia nauplii,* six times a day until 40 days post hatch (dph). We used newly hatched brine shrimp in "green conditions" (nanochloropsis paste). After 41 dph, fish were fed only brine shrimp, until they reached an approximate length of 15–20 mm standard length. Fish were then euthanized with an overdose of MS-222, and preserved in 95 % ETOH for otolith analysis (all treatments) and 10 % buffered formalin for morphological condition metrics (control and high-concentration treatments only).

We measured fish, from the high-concentration treatment (n=30) and the control treatment (n=30), to examine the effects of strontium concentration treatments on larval condition (at hatch), larval total length, oil globule volume, yolk volume and eye diameter. We excluded the low-concentration treatment due to preservation problems. Larvae were digitally imaged with an Olympus E300 dissecting microscope and a Pixelink A620 digital camera, and measurements were made using Image Pro 6.0 ® (Media Cybernetics, Silver Spring, MD).

Otolith processing and analysis

Sagittal otoliths were dissected with Teflon-coated fine forceps and glass dissecting needles. Care was taken to photograph and analyze only the sagittal otoliths in larvae, which were identified by orientation

to the notochord. The otoliths were mounted in cyanoacrylic glue for micro-chemical analysis (left otoliths were analyzed for trace elements; right otoliths for strontium isotope ratios) to minimize contamination from mounting media for chemical measurements. Otoliths were sanded with 1200 grit wet/dry sandpapers and polished with 0.3-micron alumina and a polishing cloth to remove excess glue and expose the surface of the otolith. Lastly, for chemical analysis, the otoliths were washed with 1 M nitric acid for 5 to 10 s, rinsed in an ultrasonic water-bath for 5 min, and dried under a glass 100-laminar flow-hood.

Elemental concentrations of strontium (^{88}Sr) (N= 252 individuals) were analyzed with laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) (Agilent 7200a quadrapole ICP-MS, coupled with a Nd:YAG, New Wave, UP 213 laser ablation) at the UC Davis Interdisciplinary Center for Plasma Mass Spectrometry using methods developed in Hobbs et al. 2007. A single spot on the core of the otolith was quantified at a diameter of 20 μm (~2× diameter of core and approximately 3 days post hatch), with the laser pulsing at 5 Hz, for a dwell time of 30 s. Spot size was determined based on minimizing the standard error of NIST 612 glass standard while also minimizing the spot diameter. Data were collected in a time-resolve mode for a total of 90 s. Background gas signal was collected for 30 s prior to the sample signal. The isotope of interest ^{88}Sr, was background corrected, calibrated to an internal standard (^{45}Ca), and standardized to known concentrations using NIST 612 glass standard (National Institute of Standards and Technology - U.S. Department of Commerce).

Otolith strontium isotope ratios (^{87}Sr:^{86}Sr) (N=123 individuals) were measured with a multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) (*Nu Plasma HR* from Nu Instrument Inc.) interfaced with a Nd:YAG 213 nm laser (New Wave Research UP213) for strontium isotope measurement by laser ablation (Hobbs et al. 2005). A laser beam spot of 40 μm diameter (4× core diameter and approximately 10 days post hatch) was shot on the core of the otolith with the laser pulsing at 10 Hz and at 75 % power output. Spot size was determined based on minimizing the standard error of repeated measurements of a marine mollusk shell while minimizing the spot diameter. Helium carrier gas was used to maximize sensitivity and minimize sample deposition at the ablation site, and was mixed with Argon gas between the laser sample cell and the plasma source. Gas blank and background signals were monitored until ^{84}Kr and ^{86}Kr stabilized after the sample change (i.e. exposing sample cell to the air) and were measured for 30 s. The laser was typically fired for 90–120 s, and background signals were subtracted from the measured signals automatically. Strontium isotope ratios (^{87}Sr:^{86}Sr) were normalized relative to ^{86}Sr:^{88}Sr (0.1194) to correct for instrumental mass fractionation. Mass 85 was monitored to correct for any ^{87}Rb interference on ^{87}Sr. Analytical precision was determined by using the results of replicate analyses of a modern marine mollusk at the beginning and end of several analytical sessions. Replicate analyses of this mollusk with a laser beam spot of 40 μm diameter have yielded ^{87}Sr:^{86}Sr=0.70919±0.000096 (n=28), consistent with modern seawater values of 0.70918. Data reduction was conducted off-line in Matlab 9.0.

Statistical analysis

To determine statistical significance of experimental peritoneal injection treatments, we used One-Way Fixed Effects Analysis of Variance (ANOVA), with three levels in the treatment effect (control, low and high-concentrations) with response variables strontium (^{88}Sr) concentration and separate analyses for strontium isotope ratios ^{87}Sr:^{86}Sr and growth rate. To compare morphometric characteristics of treatment groups (control and high-concentration), we used a t-test. All data were explored for normality prior to analysis and found to fit a normal distribution with a Shapiro-Wilks test. Statistical significance was

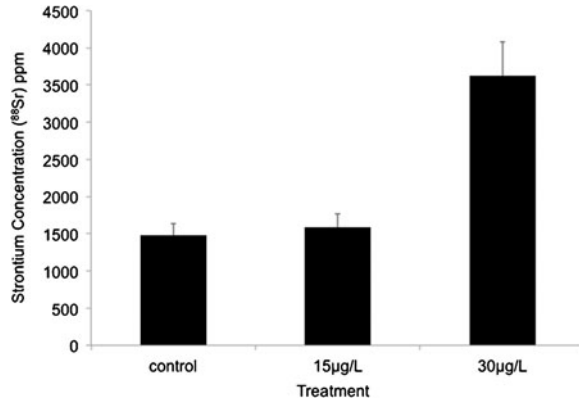

Fig. 1 Plot of mean of ^{88}Sr concentrations from each of three treatments control saline solution (1‰), a low Sr dose 15 μg/L and high Sr dose 30 μg/L. *Error bars* are ± 1 se

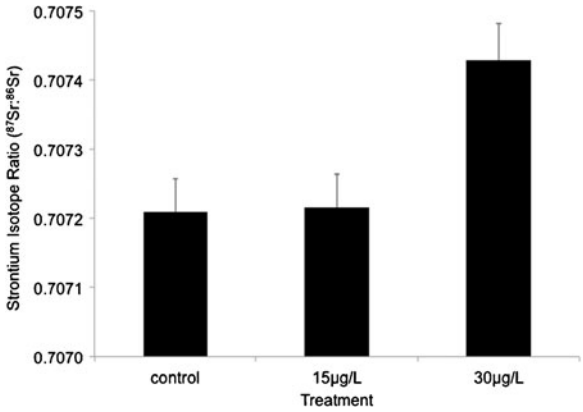

Fig. 2 Plot of mean ± 2σ of ^{87}Sr:^{86}Sr ratios from each of three treatments (control 1 % saline solution, a low Sr dose 15 µg/L and high Sr dose 30 µg/L. *Error bars* are ± 1 se

accepted at an alpha of 0.05. All statistical analyses were conducted in Systat 10.0.

Results

Peritoneal injection of strontium chloride hexahydrate proved successful in artificially marking the otoliths of delta smelt offspring. The high-strontium concentration treatment (30 µg/L) exhibited significantly elevated concentrations of strontium ^{88}Sr in the otolith cores, relative to the low-strontium concentration (15 µg/L) and the saline control (one-way ANOVA, $F_{2,249}=59.5$, $p<0.001$). However, the low-strontium concentration (15 µg/L) was not significantly different from the control (Fig. 1). The high-strontium concentration treatment also resulted in significantly elevated ^{87}Sr:^{86}Sr ratios, relative to the low-concentration and the saline control (one-way ANOVA, $F_{2,120}=41.4$, $p<0.001$). Again, the low-strontium concentration and control were not notably different from each other (Fig. 2).

Larval condition at hatch was affected by the high-strontium concentration (Fig. 3a-d). The size of the oil globule and yolk was significantly reduced for fish from the high-strontium concentration treatment compared to the controls (*note: the low-concentration was not preserved for morphological condition metrics*). However, fish length and eye diameter were not significantly different (Table 1). Larval growth rates for the 65 days of rearing were significantly different among the three treatments (one-way ANOVA, $F_{2,168}=48.1$, $p<$

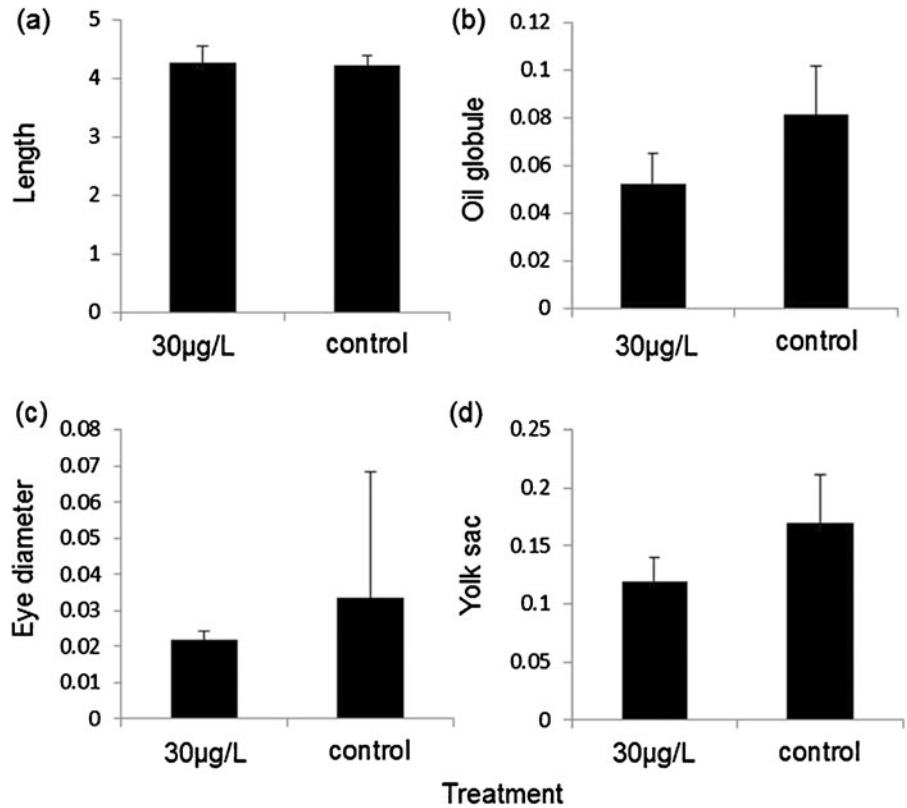

Fig. 3 Morphometric measurements of newly hatched larvae ($n=30$) from the control and high-dose treatments. **a** standard length (mm); **b** oil globule area (mm^2); **c** eye diameter (mm^2); **d** yolk sac area (mm^2). All *error bars* are ± 1 sd

Table 1 Students' *t*-test statistic for morphometrics of control and 30 μg/L test fish

	t	df	p
Length	−0.453	32	0.654
Eye	1.468	18	0.159
Oil Globule	5.201	30	<0.001
Yolk	4.844	26	<0.001

0.001), with fish from the high-concentration exhibiting slower growth (Fig. 4).

Discussion

Peritoneal injection of strontium chloride hexahydrate into ripe female fish proved successful for marking offspring. A strontium concentration of 30 μg/L resulted in elevated strontium concentrations (^{88}Sr) and strontium isotope ratios (^{87}Sr:^{86}Sr) in otolith cores of offspring, although our lower concentration 15 μg/L did not result in a tag distinguishable from controls. Previous studies have successfully marked offspring through peritoneal injections of an artificially "enriched" isotope of barium (^{137}Ba) or strontium (^{86}Sr) (Thorrold et al. 2006; Munro et al. 2009). The use of enriched or "spiked" isotopes in the marking of fish can be expensive and problematic for species that may be consumed or are endangered, as in this study. Our study utilized strontium chloride hexahydrate, a less expensive alternative to using enriched isotope solutions. Shippentower et al. (2011), similarly utilized strontium chloride hexahydrate to transgenerationally mark steelhead offspring. We also found strontium chloride to provide an elevated strontium mark on offspring otolith cores, however we also found strontium chloride hexahydrate to provide a unique strontium isotope ratio (^{87}Sr:^{86}Sr), providing an additional chemical mark.

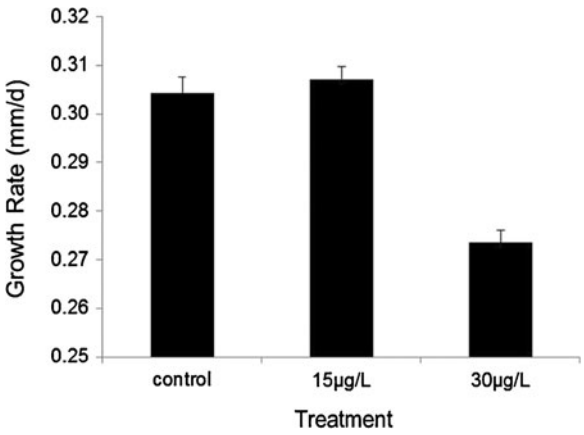

Fig. 4 Growth rate (mm/d) of experiment treatment delta smelt at 65 days post hatch. *Error bars* are ± 1 se

For transgenerational tagging to be a reliable means to mark offspring for experimental or stock enhancement purposes the effects on offspring condition and health are important. We found that the high-concentration treatment did not affect the length of larvae at hatch, but did have adverse effects on yolk and oil reserves at hatch. Moreover, growth rates of the high-concentration treatment were reduced relative to the low-concentration and control treatments. Numerous studies have quantified the link between energy reserves of larvae and subsequent growth rates. Furthermore growth in the early life has been demonstrated to have a profound effect on survival and recruitment to adulthood (Houde 1987; Meekan and Fortier 1996; Hare and Cowen 1997; Bergenius et al. 2002). Although we did not directly quantify lab survival in this study, similar numbers of offspring from each treatment remained at the end of the study, thus it was not clear whether the effects we observed ultimately translated into reduced survival.

The disadvantage of using this technique relative to enriched isotope chemical tags is that much larger dosages are required to create a chemical mark unique to natural levels, which can have adverse effects on offspring condition. Enriched isotope chemical markers are more effective at lower dosages and have been shown to have little or no adverse effects on offspring (Munro et al. 2009). Furthermore, Williamson et al. (2009) found enriched barium isotope exposures to dissipate in muscle tissue of adults in only a few weeks, thus the use of enriched isotopes may be warranted for the use of fish that may be consumed by humans. Ultimately tradeoffs do exist in using strontium chloride, a simple and relatively inexpensive chemical versus enriched isotope solution, which may be expensive or difficult to administer to consumed, or endangered fishes.

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References

Almany GR, Berumen ML, Thorrold SR, Planes S, Jones GP (2007) Local replenishment of coral reef fish populations in a marine reserve. Science 316:742–744

Bergenius MA, Meekan MG, Robertson DR, McCormick MI (2002) Larval growth predicts the recruitment success of coral reef fish. Oecologia 131:521–525

Brothers EB (1990) Otolith Marking. Pages 182–202 in Parker NC, Giorgi AE, Heidinger RC, Jester Jr DB, Prince ED Winans GA Editors. Fish-marking techniques. American Fisheries Society, Symposium 7, Bethesda, Maryland

Campana SE, Chouinard GA, Hanson JM, Fréchet BJ (2000) Otolith elemental fingerprints as biological tracers of fish stocks. Fish Res 46:343–357

Elsdon TS, Gillanders BM (2003) Reconstructing migratory patterns of fish based on environmental influences on otolith chemistry. Rev Fish Biol Fisher 13:219–235

Hare JA, Cowen RK (1997) Size, growth, development and survival of the planktonic larvae of *Pomatomus saltatrix* (Pisces: Pomatomidae). Ecology 78:2415–2431

Hobbs JA, Yin QZ, Burton J, Bennett WA (2005) Retrospective determination of natal habitat for an estuarine fish with otolith strontium isotope ratios. Mar Freshw Res 56:655–660

Hobbs JA, Bennett WA, Burton JE, Gras MA (2007) Classification of larval and adult delta smelt to nursery areas by use of trace elemental fingerprinting. Trans Am Fish Soc 136:518–527

Houde ED (1987) Fish early life dynamics and recruitment variability. Am Fish Soc Symp 2:17–29

Jones GP, Milicich MJ, Emslie MJ, Lunow C (1999) Self-recruitment in a coral reef fish population. Nature 402:802–804

Jones GP, Planes S, Thorrold SR (2005) Coral reef fish larvae settle close to home. Curr Biol 15:1314–1318. doi:10.1016/j.cub.2005.06.061

Kuroki M, Buckley RM, LeClair LL, Hauser L (2010) Validation and efficacy of transgenerational mass marking of otoliths in viviparous fish larvae. J Fish Biol 77:292–298

Meekan MG, Fortier L (1996) Selection for fast growth during the larval life of Atlantic cod *Gadus morhua* on the Scotia Shelf. Mar Ecol Prog Ser 137:25–37

Munro AR, Gillanders BM, Elsdon TS, Crook DA, Sanger AC (2008) Enriched stable isotope marking of juvenile golden perch (Macquaria ambigua) otoliths. Can J Fish Aquat Sci 65:276–285. doi:10.1139/F08-010

Munro AR, Gillanders BM, Thurstan S, Crook DA, Sanger AC (2009) Transgenerational marking of freshwater fishes with enriched stable isotopes: a tool for fisheries management. J Fish Biol 75:668–684. doi:10.1111/j.1095-8649.2009.02352.x

Nielsen LA (1992) Methods of marking fish and shellfish. American Fisheries Society, Special Publication 23, Bethesda, Maryland

Schroeder SL, Knudsen CM, Volk EC (1995) Marking salmon fry with strontium chloride solutions. Can J Fish Aquat Sci 52:1141–1149

Secor DH, White MG, Dean JM (1991) Immersion marking of larval and juvenile hatchery-produced striped bass with oxytetracycline. Trans Am Fish Soc 120:261–266

Secor DH, Henderson-Arzapalo A, Piccoli PM (1995) Can otolith microchemistry chart patterns of migration and habitat utilization in anadromous fishes? J Exp Mar Biol Ecol 192:15–33

Secor DH, Rooker JA, Zlokovitz E, Zdanowicz VS (2001) Identification of riverine, estuarine, and coastal contingents of Hudson River striped bass based upon otolith elemental fingerprints. Mar Ecol Prog Ser 211:245–253

Shippentower GE, Schreck CB, Heppell SA (2011) Who's your momma? Recognizing maternal origin of juvenile steelhead using injections of strontium chloride to create transgenerational marks. Tran Am Fish Soc 140:1330–1339

Swearer SE, Shima JS, Hellberg ME, Thorrold, SR, Jones GP, Robertson DR, Morgan SG, Selkoe KA, Ruiz GM (2002) Evidence of self-recruitment in demersal marine populations. Bull Mar Sci 70(suppl 1):251–271

Thorrold SR, Jones GP, Hellberg ME, Burton RS, Swearer SE, Neigel JE, Morgan SG, Warner RR (2002) Quantifying larval retention and connectivity in marine populations with artificial and natural markers. Bull Mar Sci 70(suppl 1):291–308

Thorrold SR, Jones GP, Planes S, Hare JA (2006) Transgenerational marking of embryonic otoliths in marine fishes using barium stable isotopes. Can J Fish Aquat Sci 63:1193–1197

Tsukamoto K (1985) Mass marking of ayu plecoglossus-altivelis eggs and larvae by tetracycline-tagging of otoliths. Nip Suis Gak 51(6):903–912

Tsukamoto K, Kuwada H, Hirokawa J, Oya M, Sekiya S, Fujimoto H, Imaizumi K (1989) Size-dependent mortality of red sea bream, *pagarus major*, juveniles released with fluorescent otolith-tags in News Bay, Japan. J Fish Biol 35:59–69

Williamson DH, Jones GP, Thorrold SR, Frisch AJ (2009) Transgenerational marking of marine fish larvae: stable isotope retention, physiological effects and health issues. J Fish Biol 74:891–905. doi:10.1111/j.1095-8649.2008.02176.x